Histochem Cell Biol (2007) 127:463–472
DOI 10.1007/s00418-007-0274-x

ORIGINAL PAPER

Four-dimensional imaging of filter-grown polarized epithelial cells

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Accepted: 21 January 2007 / Published online: 17 February 2007

Abstract Understanding how epithelial cells generate and maintain polarity and function requires live cell imaging. In order for cells to become fully polarized, it is necessary to grow them on a permeable membrane filter; however, the translucent filter obstructs the microscope light path required for quantitative live cell imaging. Alternatively, the membrane filter may be excised but this eliminates selective access to apical and basolateral surfaces. Conversely, epithelial cells cultured directly on glass exhibit different phenotypes and functions from filter grown cells. Here, we describe a new method for culturing polarized epithelial cells on a Transwell® filter insert that allows superior live cell imaging with spatial and temporal image resolution previously unachievable using conventional methods. Cells were cultured on the underside of a filter support. Epithelial cells grown in this inverted configuration exhibit a fully polarized architecture, including the presence of functional tight junctions. This new culturing system permits four-dimensional (three spatial dimension over time) imaging of endosome and Golgi apparatus dynamics, and permits selective manipulation of the apical and basolateral surfaces. This new technique has wide applicability for visualization and manipulation of polarized epithelial cells.

Keywords Four-dimensional imaging · Live cell imaging · Polarized epithelial cells · Permeable filter insert · MDCK cells

Electronic supplementary material The online version of this article (doi:10.1007/s00418-007-0274-x) contains supplementary material, which is available to authorized users.

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Introduction

Partitioning of the plasma membrane into distinct apical and basolateral membrane surfaces and subsequent formation of an electrically resistant epithelial monolayer by tight junctions are characteristic features of polarized epithelial cells. Tight regulation of membrane and protein trafficking pathways is necessary to ensure that proteins and lipids are correctly sorted to organelles and the apical and basolateral surfaces (Simons and Fuller 1985; Matter and Mellman 1994; Mostov et al. 2003; Rodriguez-Boulan et al. 2005). Proper sorting and function of epithelial cells are closely linked to and dependent on attaining full polarization. Whereas much is known about how and where protein and lipid components target within epithelial cells, the specific pathways these components follow and the dynamics of the intracellular compartments they traverse remain undefined due to lack of suitable live cell imaging approaches.

Studies of epithelial transport utilize permeable membrane filter supports for cell culture (Misfeldt et al. 1976; Cereijido et al. 1978). Full polarization is achieved when cells establish and maintain distinct

extracellular environments facing their apical and basolateral surfaces. A polarized monolayer of cells grown on such supports exhibits all characteristics of epithelial cells in tissue, including formation of circumferential intercellular occluding junctions with selective permeability characteristics and electrical resistance (Misfeldt et al. 1976; Cereijido et al. 1978; Richardson and Simmons 1979). Nevertheless, live cell imaging of these cells is difficult due to the intervening translucent polyester membrane, which prevents direct observation by an inverted microscope. Attempts to overcome this limitation include growth of epithelial cells on glass (Kreitzer et al. 2003). However, cells grown on glass polarize less well than do cells grown on filters, which provide a permeable platform that allows apical and basolateral surfaces to face different extra cellular environments. Alternatively, for direct microscopy observation, the polyester membrane, which contains a monolayer of cells can be excised and inverted directly onto a glass slide (Hua et al. 2006; Paladino et al. 2006). This approach is cumbersome and can introduce artifacts such as loss of functional polarization. The inverted culture system overcomes these problems. Other investigators have described systems using inverted MDCK cells in culture. These systems retain the epithelial monolayer and permit live cell imaging; however, each involves custom modification of Millipore® filters either creating a cup using additional filter units or parafilm, and/or removing the legs of the filter unit (Rosenberg et al. 1991; Wang et al. 2000).

The procedure described in this report uses pre-existing commercially available Transwell® filter inserts without custom modification; therefore, it is considerably simpler to perform. The filters are used without manipulation except for direct transfer to a glass chamber slide filled with liquid medium. Cells are observed using an inverted microscope with the possibility of separately or individually accessing apical and basolateral surfaces. Using fluorescence imaging approaches, we compared this new method with the conventional method of culturing polarized epithelial cells on the inside of a filter insert, and show that there is no difference in the morphology and function of both epithelial monolayers. Live imaging of polarized cells cultured on the filter underside provided unprecedented resolution of dynamics of early endosomes and the Golgi apparatus without perturbing the monolayer. Imaging also permitted visualization of endocytic tracer uptake when added to either apical or basolateral membranes. We conclude that the underside filter-culturing method has wide applicability to four-dimensional (4D) live cell imaging of fully polarized cells grown on permeable filter supports.

Materials and methods

Cell culture

MDCK II cells were maintained in MEM supplemented with 4 mM L-glutamine and 10% FBS at 37°C with 5% CO_2 levels. To culture cells on the underside of a Transwell® insert (Fig. 1a), a 12 mm permeable filter with a 0.4 μm pore size (Corning, Acton, MA, USA) was placed upside-down inside the chamber of a 6-well tissue culture plate. A total of 2.26×10^4 cells in a 100 μl-volume were seeded on top of the filter and the 6-well plate cover was placed against the top of the filter to prevent evaporation. Cells were allowed to adhere for 5 h prior to re-inversion of the Transwell® permeable insert, and subsequently cultured for 5 days. To culture cells in the conventional configuration (Fig. 1a), 2.26×10^4 cells were seeded directly into the 12 mm filter insert and allowed to grow for 5 days as previously described (Bacallao et al. 1989). In both types of culture, medium was replaced daily.

Measurement of transepithelial electric resistance (TER)

Transepithelial electric resistance values were measured using a Millicell-ERS voltohmmeter (Millipore, Billerca, MA, USA) according to the manufacturer's instructions. The same set of filter inserts was measured throughout to follow the change in TER.

Plasmids

The plasmid constructs syntaxin 3-GFP and VAMP 8-GFP were kind gifts from Roberto Weigert (NHLBI, NIH, Bethesda, MD, USA), and NTCP-GFP from Frederick J. Suchy (Mount Sinai School of Medicine, New York, NY, USA). GalT-mRFP was constructed by exchanging GFP (Nichols et al. 2001) for monomeric red fluorescent protein (mRFP). Cell transfection was performed using either Lipofectamine Plus or Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions.

Primary antibodies and reagents

Rabbit polyclonal antibodies against ZO-1, Claudin-2 was from ZYMED® Laboratories (Carlsbad, CA, USA), and mouse monoclonal antibodies against α-tubulin and γ-tubulin were from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal antibody against giantin was kindly provided by Adam D. Linstedt (Carnegie Mellon University, Pittsburgh, PA, USA).

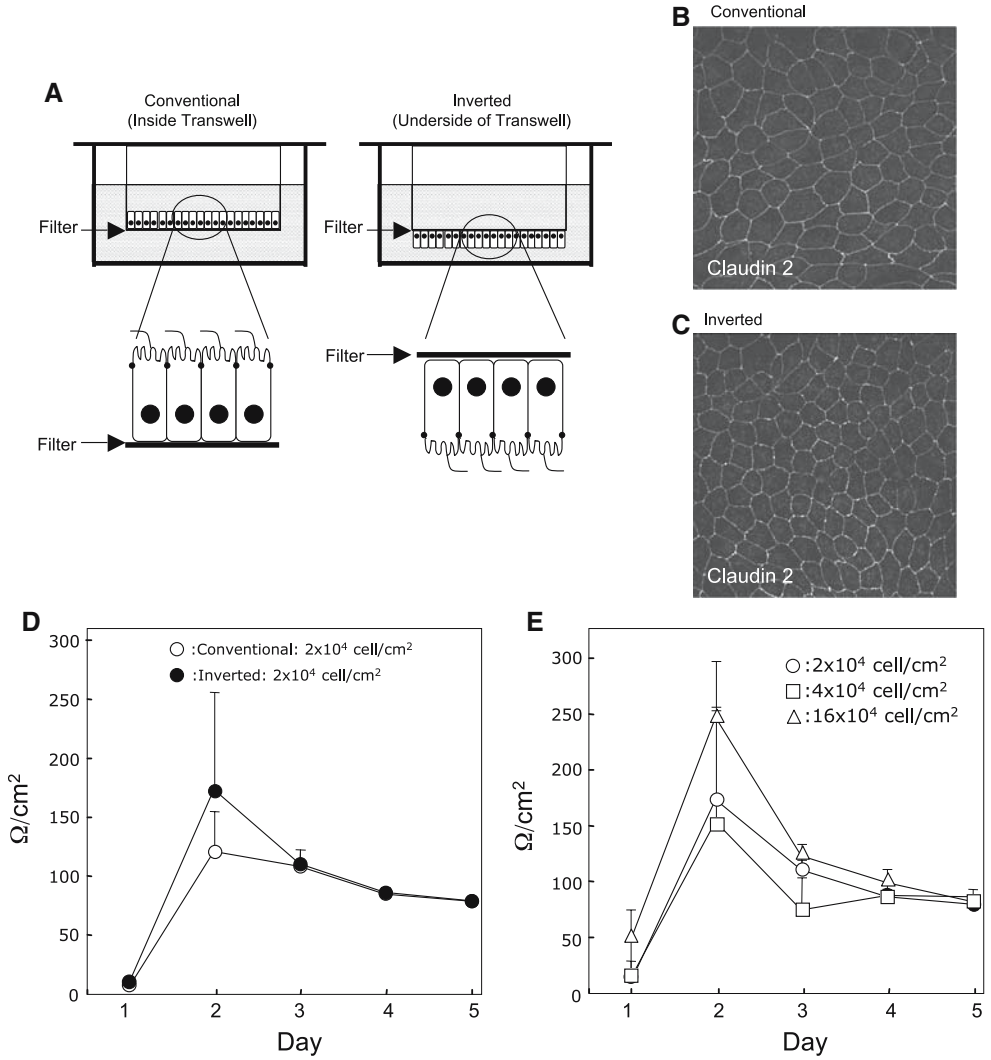

Fig. 1 Establishment of a single polarized monolayer and functional tight junctions. **a** Schematic diagram of MDCK cells grown inside (conventional) and on the underside (inverted) of a filter insert. Claudin-2 immunostaining of MDCK cells grown in the conventional (**b**) and inverted (**c**) configurations. **d** Transepithelial electric resistance (TER) measurements of MDCK cells cultured in the conventional and inverted configurations. Mean ± SD ($n = 6$). **e** TER measurements of MDCK cells seeded at different densities on the underside of Transwell filters. Mean ± SD ($n = 6$)

FM4-64 was obtained from Molecular Probes™ (Carlsbad, CA, USA), and brefeldin A (BFA) was obtained from AG Scientific Inc (San Diego, CA, USA).

Immunofluorescence staining and confocal fluorescence imaging

Cells were fixed with methanol at 4°C for 15 min and subsequently incubated with primary antibody followed by secondary antibody conjugated to a fluorophore. The porous filter was excised and mounted onto a glass slide using Fluoromount G (Electron Microscopy Sciences, Hatfield, PA, USA).

Confocal scanning microscopy was performed on Zeiss LSM 510 confocal systems. Images were taken with a 63 × 1.4 NA Apochromat oil immersion objective lens (Carl Zeiss, Inc. Thornwood, NY, USA) with the pinhole set to 1 airy unit. Image processing was performed using Zeiss LSM 5 image examiner software. Sequential images were processed with Adobe® Photoshop® CS (Adobe Systems, San Jose, CA, USA) and Quick Time Player (Apple Computer, Cupertino, CA, USA).

Live cell imaging of the filter-grown MDCK monolayer

Live cell imaging was performed using UltraView LCI confocal system (PerkinElmer, Wellesley, MA, USA) equipped with ECLIPSE TE2000-S inverted microscope (Nikon, Melville, NY, USA). All images were

taken using a 100 × 1.4 NA oil-immersion objective lens. The monolayer grown on the underside of a permeable filter insert was placed into a 2-well Lab-Tek™ Chambered coverglass (Nalge Nunc, Rochester, NY, USA). Temperature during imaging was maintained at 37°C using an ASI 400 Air Stream Incubator (NEVTEK, Williamsville, VA, USA). For imaging with longer time points, the culture medium was topped off with mineral oil to avoid pH change and evaporation. Alternatively, CO_2 independent media was also used. Sequential images were processed using Adobe Photoshop CS, Volocity 4.0 (Improvision, Lexington, MA, USA) and Quick Time Player.

Results

Cells fully polarized in the inverted configuration

To test the suitability of culturing cells on the underside of a Transwell® permeable filter, we observed seeded MDCK cells after reinversion of the Transwell® insert. Greater than 98% of cells seeded in this fashion adhered to the filter underside and became confluent on the second or third day after plating (data not shown). MDCK cells can thus readily be cultured on the underside of a Transwell® filter.

During establishment of a polarized monolayer, MDCK cells undergo spatial remodeling of cellular architecture. Fully polarized cells are defined by several features: (a) tight junctional complexes localize approximately 10 μm above the basal surface of the cells; (b) apical and basolateral membrane proteins localize to their respective membrane domains; (c) microvilli emanate and cilia extends from the apical surface; (d) Golgi complex forms a ribbon-like structure located apically above and partially capping the nucleus; (e) the microtubule organizing center (MTOC) localizes immediately underneath the apical membrane, and (f) microtubules organize in a web-like mesh distinctly visible at the apical portion of the cell (Richardson et al. 1981; Balcarova-Stander et al. 1984; Bacallao et al. 1989). Furthermore, TER of the monolayer stabilizes to constant values during polarization.

To test for tight junction formation, MDCK cells grown for 5 days in the inverted configuration were subjected to immunostaining of tight junctional proteins, claudin-2 (Fig. 1c) and ZO-1 (Fig. 3). The observed pattern of staining was indistinguishable from that observed in MDCK cells grown in the conventional configuration (Fig. 1b).

To evaluate tight junctional integrity, TER was determined during a 5-day culture period, and compared with MDCK cells cultured conventionally. Under both configurations, peak TER values were achieved on the second day after plating and decreased thereafter (Fig. 1d), with no statistically significant differences. Cells seeded at different densities achieved maximal TER at similar times after plating on the filter underside (Fig. 1e).

Next, we investigated whether the plasma membrane of MDCK cells cultured in the inverted configuration polarized into separate apical and basolateral regions, and if so, how these domains were oriented relative to the filter surface. For this, we examined the distribution of known apical and basolateral markers in these cells. In addition, we also identified the distribution of microvilli and primary cilia, which are structural landmarks of the apical plasma membrane. The apical membrane marker, syntaxin 3-GFP (Low et al. 1996), and the basolateral membrane marker, Na^+ taurocholate cotransporting polypeptide (NTCP)-GFP(Sun et al. 2001), were stably transfected into MDCK cells grown in the inverted configuration. Upon viewing the cells under the microscope, syntaxin 3-GFP was enriched on the membrane distal to the filter surface (Fig. 2a), whereas NTCP-GFP was localized adjacent to the filter. Other apical membrane markers, including glycosyl phosphatidylinositol-anchored protein (GPI)-GFP (Nichols et al. 2001), p75-GFP (Kreitzer et al. 2000) and bile salt export pump-YFP (Wakabayashi et al. 2004) were also distributed on the membrane distal to the filter, while another basolateral membrane marker, vesicular stomatitis virus G-CFP(Presley et al. 1997), was localized on the membrane immediately adjacent to the filter (data not shown). Scanning electron microscopy revealed that the plasma membrane distal to the filter surface had abundant microvilli and cilia (Fig. 2c), indicating that it represented the apical surface of the cell. These data showed that MDCK cells grown in the inverted configuration can properly sort apical and basolateral markers to their respective domains. Furthermore, the cells were oriented with apical membrane distal to the filter and basolateral membrane adjacent to the filter, which is the same orientation as cells grown in the conventional manner on filters (Balcarova-Stander et al. 1984; Fuller et al. 1984; Bacallao et al. 1989), albeit in an upside-down manner.

The distribution of intracellular structures in MDCK cells grown in the inverted configuration was determined by confocal fluorescence microscopy. Staining for nuclei revealed this organelle to be located near the basal membrane (Fig. 3). The Golgi apparatus, labeled using anti-Giantin antibodies, was located apically and resided in a cytoplasmic dome that partially capped the nuclei (Fig. 3a–e). Tight junctions,

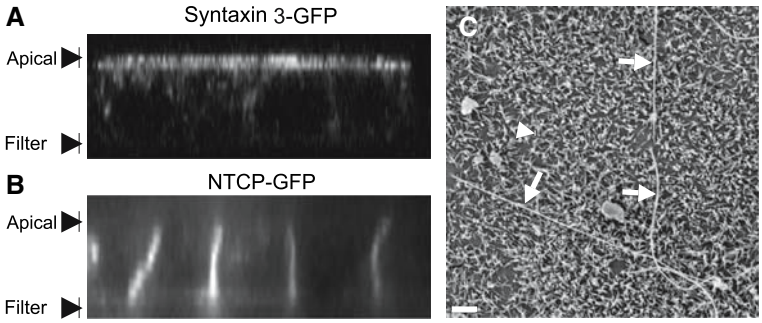

Fig. 2 Establishment of apical and basolateral membrane polarity. MDCK cells stably expressing syntaxin 3-GFP (**a**) and NTCP-GFP (**b**) cultured on the underside of Transwell filter. **c** SEM micrographs of cilia (*arrow*) and microvilli (*arrow head*) on the apical surface of monolayer grown on the underside of a filter insert. *Bar*, 200 nm

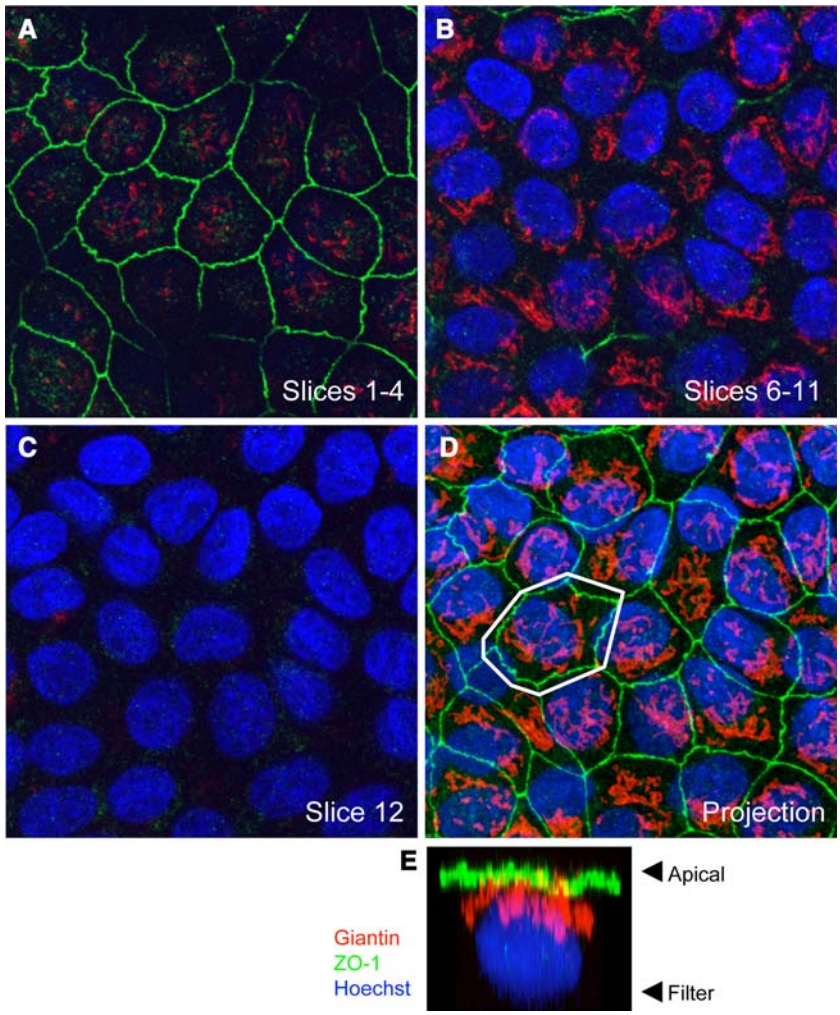

Fig. 3 Asymmetrical localization of intracellular structures. **a–d** Immunostaining of monolayer with anti giantin antibody (*red*), anti ZO-1 antibody (*green*) and Hoechst (*blue*). *White line* demarcates cell shown in X–Z projection (**e**). **f–i** The immunostaining of monolayer with anti γ-tubulin antibody (*green*), anti α-tubulin antibody (*red*) and Hoechst (*blue*). *White* demarcates cell shown in X–Z projection (**j**)

labeled using anti-ZO-1 antibodies, were located apically on the lateral membrane above the line of the Golgi apparatus (Fig. 3a–e). The microtubule-organizing center, labeled using anti-γ tubulin antibodies, was localized immediately under the apical membrane (Fig. 3f–j). Microtubules, labeled using anti-α-tubulin antibodies, were enriched at the apical portion of cytosol above the nucleus (Fig. 3f–j). The overall height of the cells was 14–17 μm. These features are similar to those found in fully polarized MDCK cells grown in the conventional manner (Bacallao et al. 1989), indicating the underside filter-culturing method does not perturb the arrangement and function of intracellular organelles.

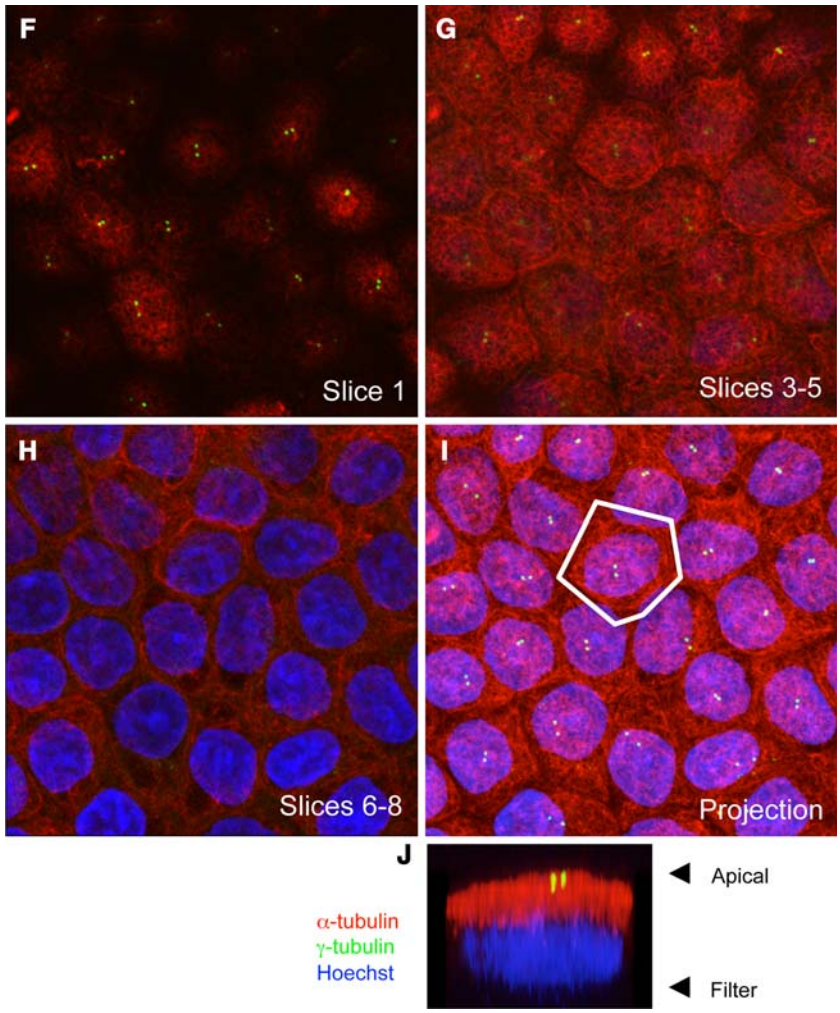

Fig. 3 continued

Live cell imaging of endosome dynamics

The standard procedure for imaging conventionally grown MDCK cells involves fixation of cells on the porous membrane film of the filter insert, subsequent excision, and mounting of the membrane film onto glass slides for imaging. An alternate procedure eliminates the fixation step before excision and involves placing the film adjacent to a coverslip with some liquid medium for viewing. Both protocols are cumbersome and manipulation of the filter can lead to tight junctional disruption and other morphological and physical artifacts.

To demonstrate that MDCK cells grown in an inverted configuration overcome these limitations and are suitable for high-resolution imaging, we cultured MDCK cells stably transfected with vesicle-associated membrane protein 8 (VAMP 8)—GFP (Steegmaier et al. 2000) in either the inverted or conventional configuration for 5 days. For imaging, the cells grown in the inverted configuration were simply placed into a Lab-Tek™ Chambered coverglass filled with culture medium. In contrast, cells grown in the conventional method were fixed, and the polyester membrane was excised and transferred to a glass slide.

Live cell imaging of MDCK cells grown in the inverted configuration revealed many different sizes and shapes of VAMP 8-GFP-labeled endosomes (Fig. 4c, Supplemental movies 1, 2). Labeled endosomes were highly dynamic and underwent numerous 'kiss and run' interactions (Duclos et al. 2000) (Supplemental movie 2). Examination of cells at different z planes revealed that the apical section of the cell was enriched in these structures. Images of fixed MDCK cells grown in the conventional method revealed only a homogeneous population of VAMP 8-GFP-positive structures with similar sizes and shapes (Fig. 4e, Supplemental movie 3). The fine tubular elements emanating from endosomes seen in live imaging using the inverted configuration were not found, presumably because of the required fixation step. These results demonstrate that culturing of polarized cells in the inverted configuration is easy and allows visualization of morphological details that are lost by fixation.

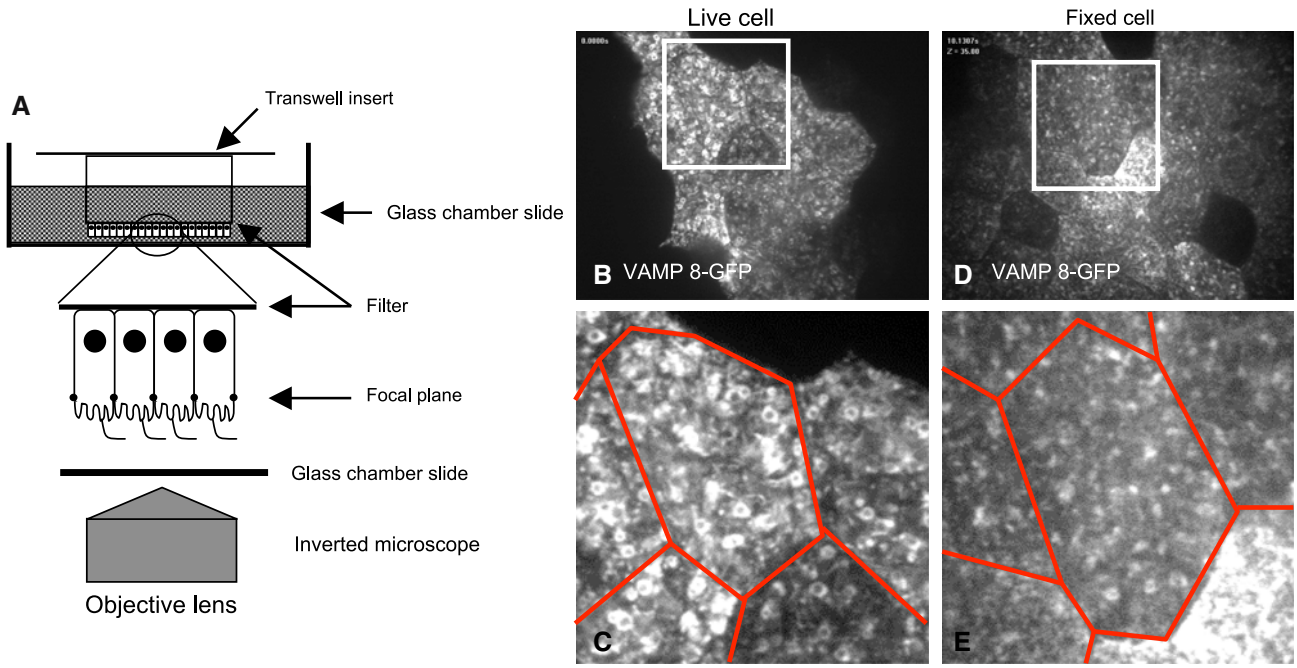

Fig. 4 Live cell imaging of endosome dynamics. **a** Schematic diagram of live cell imaging of MDCK cells grown on the underside of a filter insert. MDCK cells stably expressing VAMP 8-GFP were imaged under live conditions (**b**) and fixed conditions (**d**). **c** *Insert* in **b**. **e** *Insert* in **d**. *Red line* denotes cell boundary. See supplementary movies 1–3

Selective loading of reagents

Because the thin membrane film does not need to be excised from the Transwell® filter insert, MDCK cells grown in the inverted configuration can be immediately viewed live during and after treatment to either the apical or the basolateral membrane. To demonstrate this feature, fully polarized MDCK cells were transiently co-transfected with GPI-GFP to label the apical plasma membrane and galactosyltransferase II-mRFP (GalT-mRFP) to label the Golgi apparatus. Cells were then incubated overnight at 37°C. Live cell imaging was performed using an inverted confocal microscope. A solution of the protease, trypsin (0.05%), supplemented with calcium and magnesium was applied for 15 min either basolaterally (inside the Transwell® filter) or apically (inside the Lab-Tek™ Chambered coverglass but outside the Transwell® filter) to cleave off proteins on these surfaces (Fig. 5a). Apically applied trypsin digested GPI-GFP localized on the apical surface of living MDCK monolayer (Fig. 5b, c), whereas basolaterally-applied trypsin had no effect on this pool (Fig. 5d, e). Both treatments did not affected morphology of the Golgi apparatus (Fig. 5c, e) or the TER values (data not shown).

To demonstrate further the ease of applying reagents selectively to apical or basolateral membranes in cells grown in the inverted configuration, we made use of the lipophilic fluorescent dye, FM4-64, which binds to the plasma membrane and undergoes endocytosis (Betz et al. 1996). FM4-64 applied to the apical surface of living MDCK cells expressing GalT-GFP on the inverted microscope stage showed labeling of microvilli, cilia and endosomes, but not Golgi and basolateral plasma membranes (Fig. 6a). Conversely, basolaterally-applied FM4-64 showed labeling of basolateral membranes and endosomes, but not Golgi and apical plasma membranes (Fig. 6b). More FM4-64 dye was endocytosed from the basolateral membrane possibly due to its larger surface area relative to the apical membrane (von Bonsdorff et al. 1985; Bomsel et al. 1989). These two examples illustrate that the inverted culture system can be used to apply reagents to the apical and/or the basolateral surfaces of polarized cells and visualize their effects.

Four-dimensional imaging of organellar dynamics

Another valuable feature of the underside, filter-culturing method is that it permits 4D (three spatial dimension over time, 4D) imaging of polarized epithelial cells. This was illustrated in polarized MDCK cells expressing the Golgi marker, GalT-GFP, which were treated with BFA (50 μM), a drug that causes the Golgi apparatus to disassemble and fragment. Before addition of BFA, the Golgi appeared as an interconnected, ribbon-like tubular

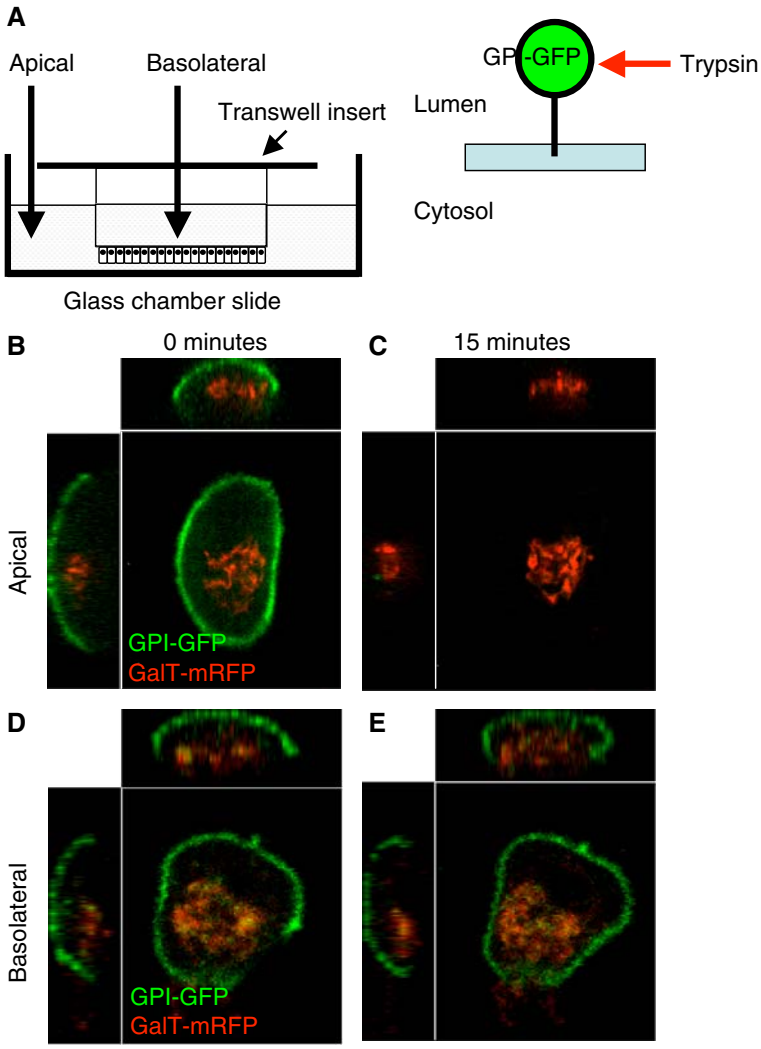

Fig. 5 Application of trypsin to specific plasma membrane domains to digest domain specific proteins. **a** Schematic diagram of reagent/drug application to specific membrane domain of the filter-grown living MDCK monolayer on the inverted microscope stages. A cell in a monolayer transiently transfected with *GPI-GFP* (*green*) and *GalT-mRFP* (*red*) before (**b**, **d**) and after trypsin application (**c**, **e**). **b**, **c** Show before and after Trypsin was applied to the apical surface, and **d**, **e** show before and after Trypsin was applied to the basolateral surface

structure (Fig. 7, Supplemental movie 4). After BFA addition to the basolateral medium, the Golgi apparatus disassembled into discrete fragments that gradually dispersed throughout the cell (Fig. 7, Supplemental movie 4). The inverted configuration is thus useful for studying organellar dynamics, such as Golgi fragmentation, in fully polarized cells.

Discussion

In this study, we described a new technique for culturing fully polarized MDCK cells on the underside of Transwell® filters which permits 4D live cell imaging. MDCK cells grown in the inverted configuration displayed similar morphological and physiological attributes as did cells conventionally grown on Transwell® filters. Cells formed a confluent polarized monolayer with a comparable transepithelial resistance property, developed tight junctions that separated apical and basolateral membrane components, and had asymmetrically distributed intracellular structures.

Previous approaches for imaging polarized monolayers of MDCK cells grown on Transwell® filters have encountered problems (Hua et al. 2006; Paladino et al. 2006). The process of excising and manipulating the flexible porous membrane film can potentially shear the confluent monolayer, leading to loss of junctional integrity between cells. Cells often appear distorted under the microscope because it is difficult to place the excised film of cells flat on the glass slide. Finally, it is impossible to apply drugs and reagents selectively to the apical or basolateral surfaces after excision. Additional problems arise depending on whether the cells are fixed or kept alive before filter excision. When cells are fixed, morphological details can be lost due to artifacts. When the cells are alive, only short-term imaging is possible because the cells no longer have distinct apical and basolateral extracellular environments, a characteristic necessary for maintaining polarity.

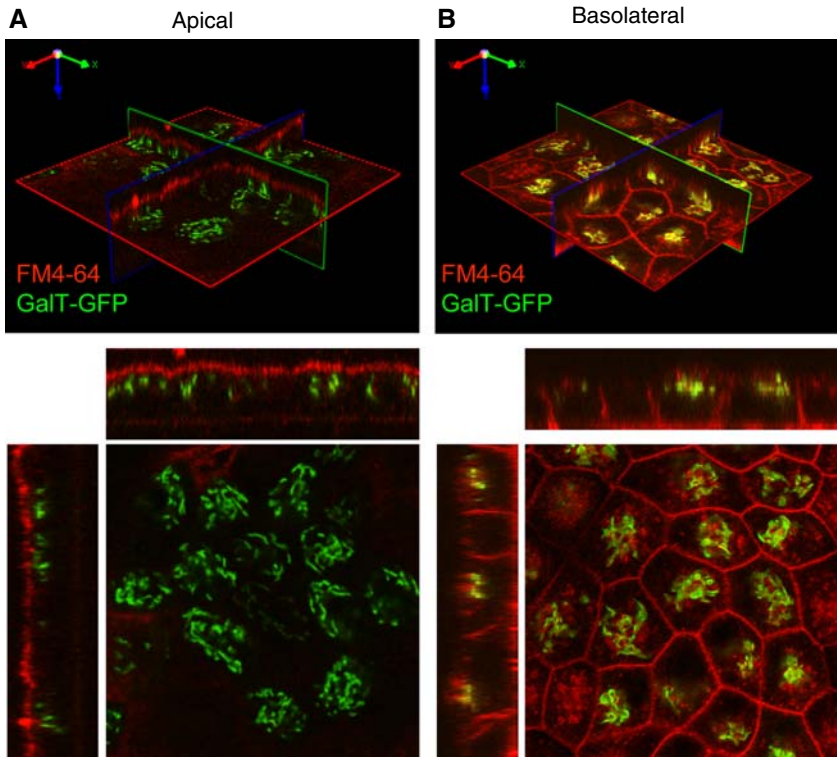

Fig. 6 Domain specific endocytosis. A living MDCK monolayer stably expressing *GalT-GFP* (*green*) was placed into a glass chamber slide and set to the inverted microscope stages. *FM4-64* lipophilic lipid dye (*red*) was applied to the apical (**a**) and basolateral (**b**) surface. After 10 min incubation, live cell imaging were subjected with inverted confocal microscope

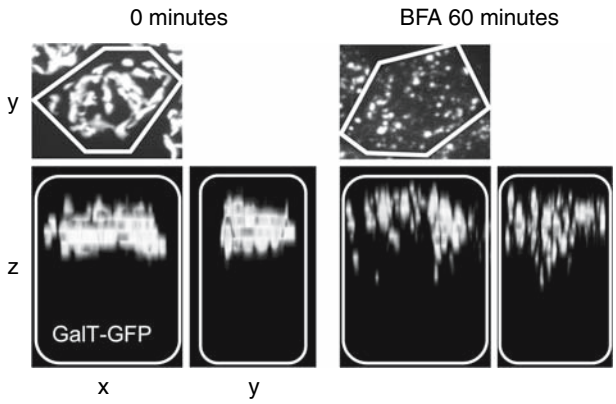

Fig. 7 Four-dimensional live cell imaging of BFA effects on Golgi. MDCK cells stably expressing *GalT-GFP* were subjected to BFA treatment at the basolateral surface. Golgi apparatus is shown before (0 min) and after (60 min) BFA treatment. See supplementary movie 4

MDCK cells grown in the inverted culture system overcome these problems. The system uses pre-existing commercially available Transwell® filter inserts; therefore there is no need for special equipment and modification. The filters are used without manipulation except direct transfer to a microscope grade glass chamber for live cell imaging. Reagents readily can be applied selectively to either apical or basolateral membrane during live cell imaging. Furthermore, 4D live cell imaging can be performed with an inverted microscope. These properties, in conjunction with improved spatial and temporal resolution of organellar dynamics, make the culturing of epithelial cells on the underside of filters a useful technique for live cell study of dynamics in fully polarized cells.

Competing interest statement

The authors declare that they have no competing financial interests.

Acknowledgments We thank Vicrotia C. Cogger for assistance with SEM; Roberto Weigert (NHLBI, NIH, Bethesda, MD, USA), Frederick J. Suchy (Mount Sinai School of Medicine, New York, NY, USA) and Adam D. Linstedt (Carnegie Mellon University, Pittsburgh, PA, USA) for providing reagents used in this study. Janet L. Larkin received support from the Oak Ridge Institute for Science and Education.

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